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Abstract

We have recently demonstrated that naphthoquinone (NQ), one of extractable chemical compounds of diesel exhaust particles (DEP), enhances antigen-related airway inflammation with goblet cell hyperplasia in mice (Inoue et al., in press). NQ, further, has enhanced lung expressions of interleukin (IL)-4 and IL-5. However, the effects of NQ on the other cardinal features of asthma have not been completely investigated. The aim of the present study was to evaluate the effects of NQ on airway responsiveness on the model. Vehicle, NQ, ovalbumin (OVA), or NQ + OVA was administered intratarcheally to ICR mice for 6 wk. Twenty four h after the last instillation, lung histology, lung function such as total respiratory system resistance (R) and Newtonian resistance (R_n), and protein level of IL-13 and mRNA level for MUC5AC in the lung were examined. Repetitive exposure to NQ aggravated antigen-related lung inflammation. NQ alone enhanced R and R_n as compared to that to vehicle without statistical significance. OVA alone or NQ plus OVA showed increases in R and R_n, which was prominent in NQ plus OVA ($p < 0.05$ vs. vehicle). Combined exposure to NQ and OVA elevated the levels of IL-13 and MUC5AC in the lung as compared with exposure to NQ or OVA alone. These results indicate that NQ can enhance airway hyperresponsiveness in the presence or absence of antigen. Also, amplified lung expressions of IL-13 and MUC5AC might contribute, partly, to the deterioration of asthma features by NQ.

Key Words: naphthoquinone, antigen, airway hyperresponsiveness, MUC5AC

Introduction

Diesel exhaust particles (DEP) have been implicated to facilitate allergic reactions (Nikasinovic et al. 2004; Takizawa 2004). *In vivo* studies from our laboratory have documented that DEP exacerbate airway inflammation induced by repetitive intratracheal instillation of antigen through the promotion of Th2 immunity (Ichinose et al. 1998; Takano et al. 1998; Takano et al. 1997).

DEP are complicated particles consisting of carbonaceous nuclei and a vast number of organic chemical compounds such as polyaromatic hydrocarbons, aliphatic hydrocarbons, heterocycles, and quinones. Among them, organic chemical components extracted from DEP reportedly augument allergic response *in vitro* (Devouassoux et al. 2002) and *in vivo* (Heo et al. 2001). Consistent with the previous reports, we have also demonstrated that extracted organic chemicals from DEP, rather than residual carbonaceous nuclei of DEP after extraction, predominantly enhance antigen-related airway inflammation in mice (Yanagisawa et al. 2006).

Otherwise, a variety of quinones have been identified as DEP components (Schuetzle 1983; Schuetzle et al. 1981). Quinones themselves have toxicological properties to serve as alkylating agents and to interact with, for example, flavoproteins to generate reactive oxygen species (ROS), which can lead to biological injury (Bolton et al. 2000; Monks et al. 1992; O'Brien 1991; Cho et al. 2004). Phenanthraquinone (PQ) is one of quinones involved in DEP (Bolton et al. 2000; Schuetzle 1983). We have recently shown that PQ itself induces lung inflammation (Hiyoshi et al. 2005a) and aggravates antigen-related airway inflammation (Hiyoshi et al. 2005b) in mice. Naphthoquinone (NQ; MW: 158) is another quinone involved in DEP (Kumagai et al. 1995; Cho et al.

2004). Detailed chemical analysis has reported that approximately 13.7 μ g of NQ is contained in 1 g of DEP (Cho et al. 2004). We have also shown that NQ deteriorates antigen-related airway inflammation in mice (Inoue et al. in press). In brief, pulmonary exposure to NQ has aggravated antigen-related airway inflammation characterized by infiltration of eosinophils and lymphocytes around the airway and an increase in goblet cells in the bronchial epithelium in a dose-dependent manner with enhanced lung expressions of Th2 cytokines such as interleukin (IL)-4 and IL-5 (Inoue et al. in press). Goblet cell hyperplasia in the airway plays a role in airway remodeling with impaired airway physiology (Cohn et al. 2004; Rogers 2004). Hence, we hypothesized that NQ could influence other pivotal hallmark of allergic asthma, i. e. airway hyperresponsiveness on the model. Furthermore, IL-13, another Th2 type cytokine, is recognized to be essential for allergic phenotypes including airway hyperresponsiveness (Grunig et al. 1998; Nakano et al. 2006; Wills-Karp et al. 1998). In addition, MUC5AC has been identified as a mucin producing gene (Morcillo and Cortijo 2006).

The present study was aimed mainly to elucidate the effects of NQ on the airway function using the same protocol as the previous study (Inoue et al. in press). We also examined the local expression of IL-13 and MUC5AC to complement mechanisms of NQ-facilitation on the model.

Materials and methods

Animals

Male ICR mice 6 to 7 wk of age and weighing 29 to 33 g (Japan Clea Co., Tokyo, Japan) were used in all experiments. They were fed a commercial diet (Japan Clea

Co.) and given water ad libitum. Mice were housed in an animal facility that was maintained at 24 to 26℃ with 55 to 75% humidity and a 12-h light/dark cycle. The studies adhered to the National Institutes of Health guidelines for the experimental use of animals. All animal studies were approved by the Institutional Review Board of National Institute for Environmental Studies.

Study protocol

Mice were divided into four experimental groups as exhibited in our recent study (shown as FIGURE 1 in the paper by Inoue et al. [in press]). The vehicle group received phosphate-buffered saline (PBS) at pH 7.4 (Nissui Pharmaceutical Co., Tokyo, Japan) containing 0.025% Tween 80 (Nacalai Tesque, Kyoto, Japan) once a week for 6 wk to serve as control. As well, the ovalbumin (OVA) group received 1 μ g of OVA (Sigma Chemical, St. Louis, MO) dissolved in the same vehicle bi-weekly and the vehicle alone another bi-weekly for totally 6 wk. Our previous study has shown that NQ (1.58 ng [0.01 nmol], 15.8 ng [0.1 nmol], 158 ng [1 nmol]/animal) dose-dependently aggravates antigen-related airway inflammation with goblet cell hyperplasia and enhanced lung expression of chemokines (macrophage chemoattractant protein-1 and keratinocyte chemoattractant: ref; Inoue et al., in press and unpublished observation). Thus, we applied the dose of NQ at 158 ng/animal, which had revealed most prominent effects, to the current experiments. The NQ group received NQ dissolved in the same vehicle every week for 6 wk. The NQ + OVA group received the combined treatment in the same protocol as the NQ and the OVA groups. In each group, vehicle, NQ, OVA, or NQ + OVA was dissolved in 0.1-ml aliquots, and inoculated intratracheally as previously described (Takano et al. 1997). The animals were studied 24 h after the last intratracheal administration.

Histologic evaluation

After exsanguinations, the lungs were fixed by intratracheal instillation with 10% neutral phosphate-buffered formalin at a pressure of 20 cm H_2O for at least 72 h. Slices 2 to 3 mm thick of all pulmonary lobes were embedded in paraffin. Sections 3 μ m thick were stained with Hematoxylin and eosin to observe and to quantitate the total numbers of polymorphonuclear cells and mononuclear cells (defined as "inflammatory cells") infiltrating to the airways. The length of the basement membrane of the airways was measured by videomicrometer (Olympus, Tokyo, Japan) in each sample slide. The number of inflammatory cells around the airways were counted with a micrometer under oil immersion. Results were expressed as the number of inflammatory cells per millimeter of basement membrane as described previously (n = 4 in each group [Takano et al. 1997]).

Analysis of lung function

In another experiment, assessment of cholinergic airway constrictor responsiveness was done with a computer-controlled small-animal ventilator (FlexiVent; Scireq, Montreal, Canada) as previously described (Card et al. 2006; Gavett et al. 1999; Kang et al. 2003; Lee et al. 2004). In brief, the mice were anesthetized with 0.1 ml per 10 g body weight of a 40 mg/ml ketamine hydrochloride given intraperitoneally. Anesthesia was maintained by supplemental administration of 30% of the initial dose at ～25-min intervals, as required. Mice were tracheostomised with a 5 mm section of metallic tubing and ventilated (FlexiVent) at 180 $b.min^{-1}$ with a tidal volume of 8 ml/kg and a positive end expiratory pressure of 2 cmH_2O.

Both the single-compartment model (using snap shot method) and the constant-phase model (using forced oscillation technique (FOT) method) of respiratory mechanics were

applied to assess lung function and airway responses to methacholine. For the single-compartment model, total respiratory system resistance (R) was determined essentially as described previously (Card et al. 2006; Gavett et al. 1999). For the constant-phase model, R_n (Newtonian resistance) was determined as described previously (Card et al., 2006; Lee et al., 2004). All data points were determined by the FlexiVent software (version 5.0) by using multiple linear regression to fit each data point to the single-compartment or the constant-phase model, as appropriate. The lung volume history of the mice was standardised prior to measurement of lung mechanics using two deep inflations. P and V data were generated by applying a 2 s sine wave volume perturbation (SW) with an amplitude of 0.2 ml and a frequency of 2.5 Hz. After 5 min of regular mechanical ventilation, the SW perturbation was applied three times and the average was taken to generate a baseline measurement. The respiratory system input impedance (Zrs) was measured during periods of apnea using a 3 s signal containing 19 mutually prime sinusoidal frequencies ranging from 0.25 to 19.625 Hz; these maneuvers generated data that were fit to the single-compartment or the constant-phase compartment models, respectively. The averages of these measurements for each mouse served as its baseline values. Following acquisition of baseline data, airway responsiveness to aerosolized methacholine (MCh: 0.125 to 50 mg/mL saline; delivered by ultrasonic nebulizer) was assessed using both snap shot and FOT methods. Aerosols were delivered for 10 s with a ventilation at 150 $b.min^{-1}$ with a tidal volume of 10 ml/kg, after which the snap shot and the FOT methods were applied consecutively every 6 s for 5 min. Peak responses during each 5-min period were determined, and only values with a coefficient of determination of 0.95 or greater were used.

Quantitation of IL-13 protein levels in the lung

In a separate series of experiments, the animals were exsanguinated and the lungs were subsequently homogenized with 10 mM potassium phosphate buffer (pH 7.4) containing 0.1 mM ethylenediaminetetraacetic acid (Sigma, St Louis MO), 0.1 mM phenylmethanesulphonyl fluoride (Nacalai Tesque, Kyoto, Japan), 1 μ M pepstatin A (Peptide Institute, Osaka, Japan), and 2 μ M leupeptin (Peptide Institute) as described previously (Takano et al. 1997). The homogenates were then centrifuged at 105,000 g for 1 h. The supernatants were stored at -80℃. Enzyme-linked immunosobent assays (ELISA) for IL-13 (R&D systems, Minneapolis, MN) in the lung tissue supernatants were conducted using matching antibody pairs according to the manufacture's instruction. The second antibodies were conjugated to horseradish peroxidase. Subtractive readings of 550 nm from the readings at 450 nm were converted to pg/ml using values obtained from standard curves generated with the limits of detection of 1.5 pg/ml (n = 7-8 in each group).

Quantitation of MUC5AC mRNA levels in the lung

In another experiment, total RNAs in the lung were extracted with ISOGEN (Nippon gene, Tokyo, Japan), according to the manufacture's instructions. cDNA synthesis were conducted according to the manufacture's protocol. The quantitation of mRNA expression was carried out by real time RT-PCR using the ABI Prism 7000 Sequence Detection System (TaqMan, Perkin-Elmer Corp., Foster City, CA), according to the manufacture's instructions. cDNAs were amplified according to the thermal profile of 50℃ for 2 min then 95℃ for 10 min, followed by up to 40 cycles at 95℃ for 15 s and 60℃ for 1 min. Specific primers and probes were obtained from Applied Biosystems. The sequences of 18S rRNA and MUC5AC, which were purchased from Perkin-Elmer, were not disclosed by the manufacturer. The quantitation of gene expression was derived from the cycle number at which the fluorescent signal crossed a threshold in the

exponential phase of the PCR reaction using the standard curve method according to the manufacturer's protocol. The relative quantitation of mRNA was normalized to an endogenous control gene (18S rRNA) (n = 6-8 in each group).

Statistical analysis

Data were reported as mean ± SEM. Differences among groups were analyzed by ANOVA followed by *Fisher's* PLSD test (Stat view version 4.0; Abacus Concepts, Inc, Berkeley, CA). Significance was assigned to *p* values smaller than 0.05.

Results

Effects of NQ on lung histology in the presence or absence of antigen

To quantitate the infiltration of inflammatory cells around the airways, we expressed the number of these cells per length of basement membrane of the airways. The number was greater in the NQ (inflammatory cells number/mm airways, mean ± SEM: 1.89 ± 0.35; N. S.) or the OVA group (5.79 ± 1.24; $P < 0.01$) than in the vehicle group (0.92 ± 0.25). The number was significantly greater in the NQ + OVA group (12.70 ± 1.38) than in the vehicle, the NQ, or the OVA group ($P < 0.01$).

Effects of NQ on lung function in the presence or absence of antigen

To evaluate the effects of NQ on MCh responsiveness, we investigated R (Fig. 1A) and R_n (Fig. 1B) in four groups of mice 24 h after the last intratracheal instillation. R was increased in each group with dose-dependency with MCh (Fig. 1A). The value was higher in the NQ, the OVA, or the NQ + OVA group than in the vehicle group, which was most prominent in the NQ + OVA group. In particular, the value in the NQ + OVA group at the MCh concentrations of 25 mg/ml ($p < 0.05$) and 50 mg/ml ($p < 0.01$)

was significantly higher than that of the vehicle group. R_n was increased in each group with dose-dependency with MCh (Fig. 1B). The value at the MCh concentration of 50 mg/ml was highest in the NQ + OVA group ($p < 0.05$ vs. the vehicle group), followed by the OVA, the NQ, and the vehicle groups.

Effects of NQ on local expression of IL-13 in the presence of antigen

To explore the role of local expression of IL-13 in the enhancing effects of NQ on antigen-related airway inflammation (Inoue et al. in press) and airway hyperresponsiveness, we quantitated protein levels of IL-13 in the lung tissue supernatants 24 h after the last intratracheal instillation (Table). The level was higher in the OVA (N. S.) or the NQ + OVA group ($p < 0.01$) than in the vehicle group. The level was more than double in the NQ + OVA group than in the OVA group, however, it did not achieve statistical significance.

Effects of NQ on local expression of MUC5AC in the presence of antigen

To investigate the effect of NQ on the expression of MUC5AC, we compared mRNA level for MUC5AC in the lung 24 h after the final intratracheal instillation (Table). The mRNA level was almost negligible in the vehicle, the NQ, or the OVA groups. The level was significantly higher in the NQ + OVA group than in the other groups ($p < 0.01$).

Discussion

Our previous study has demonstrated that NQ repeatedly administered by intratracheal route deteriorates antigen-related airway inflammation in mice, which is characterized by the infiltration of inflammatory leukocytes in both the bronchoalveolar spaces and

the lung parenchyma (Inoue et al. in press). NQ has also exaggerated antigen-induced goblet cell hyperplasia. The facilitation has been concomitant with the increased lung expression of Th2 cytokines such as IL-4 and IL-5, and chemokines such as eotaxin, macrophage chemoattractant protein-1, and keratinocyte chemoattractant. Also, NQ has exhibited adjuvant activity for the antigen-specific production of IgG_1 (Inoue et al. in press). First of all, the present study recomfirms that NQ aggravates lung inflammation related to antigen using more comprehensive morphometric analysis than that in our previous study (Inoue et al. in press). Nextly, the present study expands the previous one to show that NQ facilitates airway hyperresponsiveness in the presence or absence of antigen and lung expression of IL-13 and a mucin producing gene in the presence of antigen.

It has been documented that DEP enhance airway inflammation (Takano et al. 1997) and airway hyperreactivity (Takano et al. 1998) related to antigen. Studies about their ingredients have suggested that chemical components are attributed to the proallergic reaction of DEP (Devouassoux et al. 2002; Heo et al. 2001; Delfino 2002). We have also demonstrated that organic chemicals in DEP, rather than their carbonaceous nuclei, predominantly enhance antigen-related airway inflammation in mice (Yanagisawa et al. 2006). However, the contribution of DEP-derived components, in particular chemical ones, to the enhancement has not been fully investigated.

Quinones have also been involved as chemical components in DEP (Schuetzle 1983; Schuetzle et al. 1981). Quinones posses toxicological properties to serve as alkylating agents and to interact with, for example, flavoproteins to generate ROS, which can lead to biological injury (Bolton et al. 2000; Monks et al. 1992; O'Brien 1991; Cho et al. 2004). We have previously shown that airway exposure to PQ, one of important

quinones in DEP (Schuetzle 1983; Cho et al. 2004), induces airway inflammation, which is concomitant with lung expression of IL-5 and eotaxin *in vivo* (Hiyoshi et al. 2005a). Another study has demonstrated that PQ enhances antigen-related airway inflammation *in vivo* (Hiyoshi et al. 2005b). On the other hand, NQ, another extractable chemical compound in DEP, reportedly generates free radical, binds to thiol containing proteins, and irreversibly inactivates them (Kumagai et al. 1995). We have demonstrated that NQ also exaggerates antigen-related airway inflammation *in vivo* (Inoue et al. in press). However, whether NQ aggravates other pivotal hallmarks of asthma had not been defined. In the present study, we demonstrated that NQ moderately facilitates antigen-related airway hyperresponsiveness. However, the facilitation did not reach statistical significance as compared to antigen alone. This may be partly explained by the magnitude of airway hyperresponsiveness on the model and dose of NQ. Repetitive intratracheal exposure to antigen without preceding systemic sensitization induces less airway hyperresponsiveness (Takano et al. 1998) than typical asthma model (two intraperitoneal administration of antigen plus adjuvant with conventional antigen inhalation). As well, NQ at 158 ng/animal may be insufficient to enhance the features, although the dose of NQ can be estimated to be more than a hundred fold than that of DEP we had used in the previous studies (Takano et al. 1997; Takano et al. 1998).

In the present study, lung expression level of IL-13 was almost paralleled to that of goblet cell hyperplasia (Inoue et al. in press) and airway hyperresponsiveness related to antigen. The protein expression was greater in the NQ + OVA group than in the OVA group, however, it did not reach statistical significance. IL-13 is a key Th2 cytokine driving pathogenic changes associated with asthma. IL-13 reportedly induces/supports epithelial cell maturation and mucus secretion (Kuperman et al. 2002) and enhanced

contractility of airway smooth muscle cells (Tliba et al. 2003) *in vitro*. Also, IL-13 plays a role in airway hyperresponsivness, airway inflammation, and mucus secretion *in vivo* (Wynn 2003). Indeed, antagonism of this cytokine has been shown to prevent these asthma-related phenotypes (Kasaian et al. 2006; Wills-Karp 2004; Wynn 2003). Thus, it is possible that IL-13 partly contributed to NQ facilitation on goblet cell hyperplasia in the previous study (Inoue et al. in press) and airway hyperresponsiveness related to antigen in the present one.

Interestingly, repetitive exposure to NQ alone induced a tend toward increased R and R_n. The mechanisms of this phenomenon remain unresolved. Damaged airway epithelium results in exposure of sensory neuron and consequent airway hyperreactivity. However, it is unlikely, because our previous study has shown that NQ alone does not induce significant pulmonary epithelial damage (Inoue et al. in press). It is possible that NQ influence autonomic nerve system to lead to vagotonia-sympathicotonia imbalance. We have previously demonstrated that NQ can cause contraction of tracheal smooth muscle from guinea pig in a concentration-dependent manner via the activation of epidermal growth factor receptor (EGFR; ref Kikuno et al. 2006). Thus, it is also possible that this mechanism through EGF-EGFR pathway plays a role in this action of NQ on the current *in vivo* model. Although further investigation is needed, the present result suggests repeated (chronic) exposure to NQ could impair lung function.

Our previous report about NQ-facilitation on the asthma model has included enhancing effects on goblet cell hyperplasia (Inoue et al. in press). Of the 19 human mucin genes identified to date, MUC5AC is considered one of master genes for secreted mucin of the bronchial epithelium (Rogers 2004; Zuhdi Alimam et al. 2000). In the present study,

lung expression level of MUC5AC was markedly higher in the NQ + OVA group than in the other groups. Thus, it is likely that this enhanced expression of MUC5AC contributes, at least partly, to worsened goblet cell hyperplasia induced by antigen. On the other hand, however, it remains undefined whether enhanced lung expression of MUC5AC found in the NQ + OVA group contributes to moderate enhancing effects on airway hyperresponsiveness in that group. In another point, it is somewhat atypical that MUC5AC was not induced by OVA alone, although IL-13 was induced in the present study. IL-13 may not be positively involved in MUC5AC expression or may proced the expression in this experimental protocol.

In summary, the present study has shown that NQ can induce/enhance airway hyperresponsiveness in the presence or absence of antigen. Also, NQ amplifies lung expression of IL-13 and MUC5AC in the presence of antigen. These results suggest that environmental quinones may play, in part, a role in the DEP-toxicity against asthma hallmarks in the context of airway pathophysiology. Repeated cycles of inflammation and repair in the airway in asthma lead to pathological changes in the structure that are termed “remodeling” (Rogers 2004; Cohn et al. 2004; Davies et al. 2003). Airway remodeling includes peribronchial fibrosis with increased deposition of collagen, smooth muscle hypertrophy/hyperplasia, and mucus hypersecretion (Cohn et al. 2004; Rogers 2004). These structural changes in the airway result in irreversible airway obstruction with further hyperresponsiveness. Thus, our previous study and current one suggest that chronic NQ exposure may terminally aggravate airway remodeling.

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Table. Protein levels of interleukin (IL)-13 and mRNA levels of MUC5A in the lung.

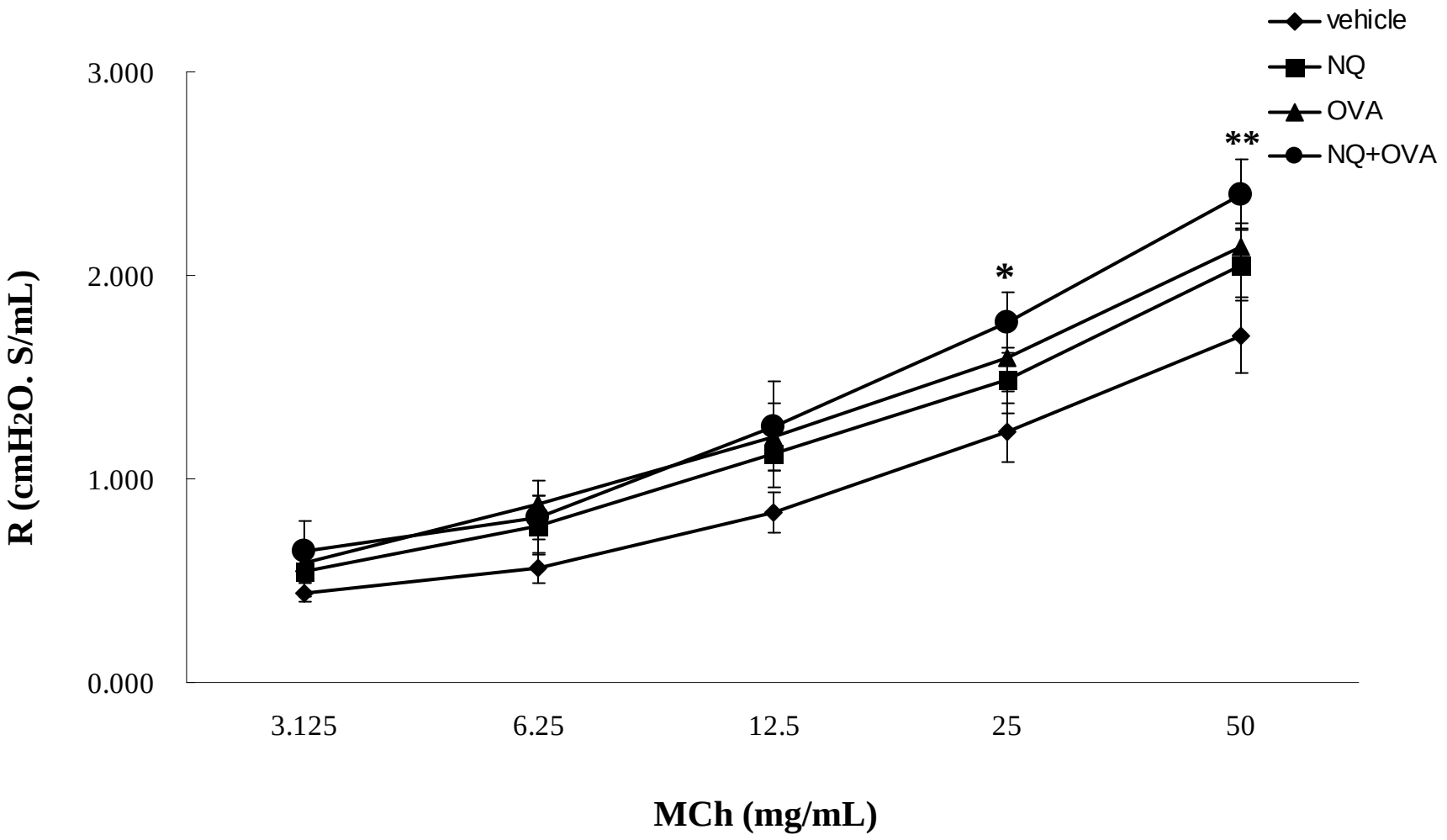
Group	IL-13	MUC5AC
	pg/total lung supernatants	relative quantitation
vehicle	0 ± 0	0.15 ± 0.05
NQ	0 ± 0	0.17 ± 0.06
OVA	44.8 ± 30.9	0.18 ± 0.02
NQ + OVA	92.2 ± 43.5 $^{*\$}$	2.24 ± 0.64 $^{*\#\$}$

Four groups of mice were intratracheally inoculated with vehicle, naphthoquinone (NQ), ovalbumin (OVA), or the combination of NQ + OVA for 6 wk. Lungs were removed and frozen 24 h after the last intratracheal administration. IL-13 protein levels in the lung tissue supernatants were analyzed using enzyme-linked immunosorbent assays (n = 7-8 in each group). Gene levels for MUC5AC were analyzed using RT-PCR (n = 6-8 in each group). Results are shown as mean ± SEM. * $P < 0.01$ vs. vehicle. $^{\$}$ $P < 0.01$ vs. NQ. $^{\#}$ $P < 0.01$ vs. OVA.

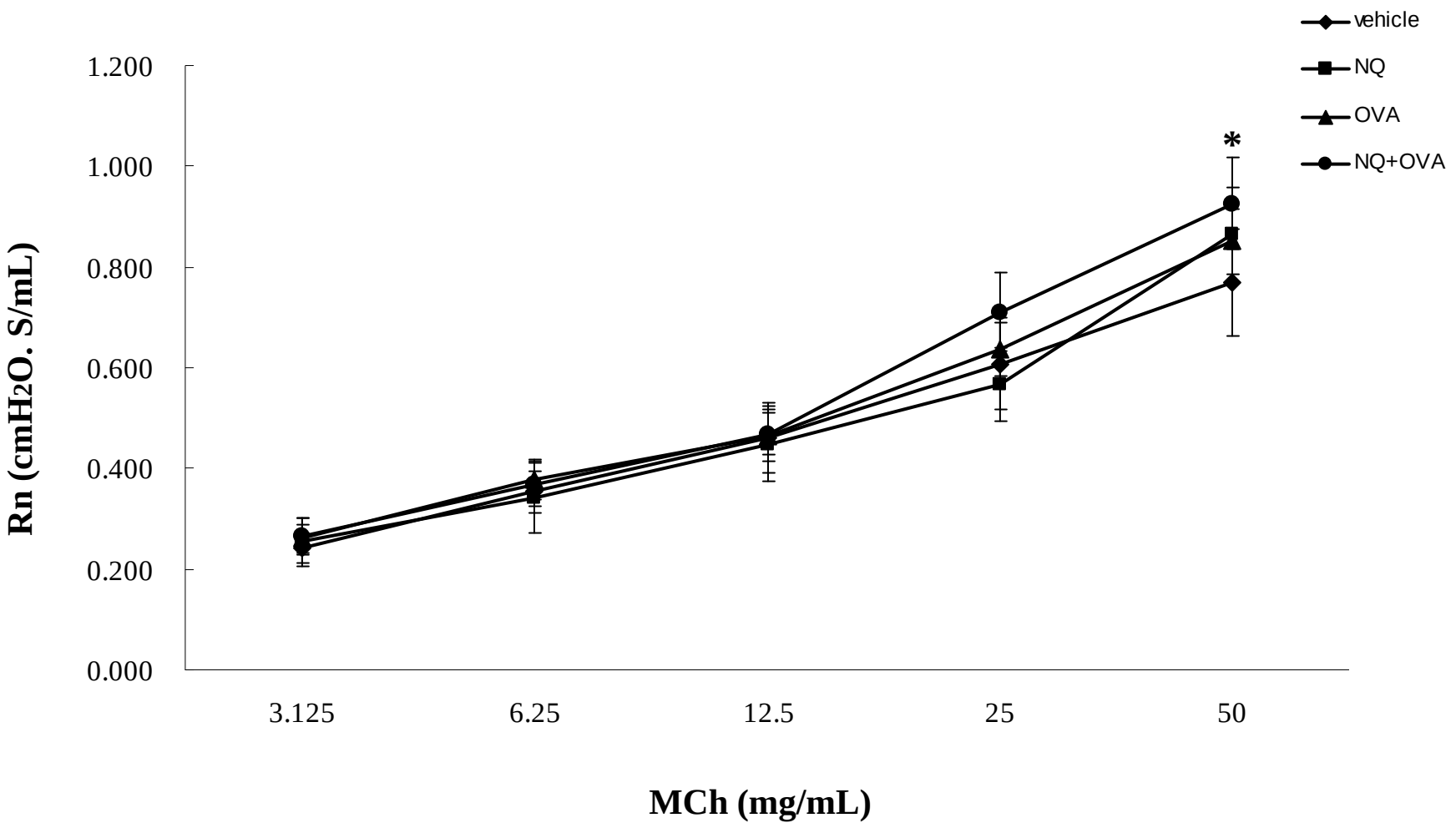
Figure Legend

Fig. 1. Dose response curve to inhaled methacholine (MCh). The animals were randomized into four experimental groups that received repeated intratracheal instillation with vehicle, naphthoquinone (NQ), ovalbumin (OVA), or the combination of NQ + OVA for 6 wk. Total respiratory system resistance (R: A) or Newtonian resistance (R_n: B) was measured 24 h after the last instillation. Data are shown as mean ± SEM (n = 10 in each group). * $P < 0.05$ vs. vehicle, ** $P < 0.01$ vs. vehicle.

A


vehicle
NQ
OVA
NQ+OVA
3.000
2.000
1.000
0.000
R (cmH2O. S/mL)
*
**
3.125
6.25
12.5
25
50
MCh (mg/mL)

B


vehicle
NQ
OVA
NQ+OVA
1.200
1.000
0.800
0.600
0.400
0.200
0.000
Rn (cmH2O. S/mL)
*
3.125
6.25
12.5
25
50
MCh (mg/mL)